Denitrification Activity of Polyphosphate Accumulating Organisms (PAOs) in Full-Scale Wastewater Treatment Plants

Ana B. Lanham[a*], Adrian Oehmen[b,f], Gilda Carvalho[b,d], Aaron M. Saunders[c], Per H. Nielsen[c], Maria A. M. Reis[b]

[a]University of Bath, Department of Chemical Engineering, Water Innovation and Research Centre, Claverton Down, BA2 7AY, Bath, UK

[b]UCIBIO, REQUIMTE, Chemistry Department FCT-UNL, 2829-516 Caparica, Portugal

[c]Department of Chemistry and Bioscience, Aalborg University, Aalborg Denmark

[d]Advanced Water Management Centre, The University of Queensland, Brisbane, QLD, 4072, Australia;

[f]School of Chemical Engineering, The University of Queensland, Brisbane, QLD, 4072, Australia

* Corresponding author: a.lanham@bath.ac.uk

ABSTRACT

A comprehensive assessment of full-scale EBPR plants (5 plants, 19 independent tests) was undertaken to determine their effectiveness in terms of aerobic and anoxic P removal. By comparing parallel P uptake tests under only aerobic or under anoxic-aerobic conditions, results revealed that introducing an anoxic stage led to an overall P removal of on average 90% of the P removed under only aerobic conditions. This was achieved with negligible higher PHA and glycogen requirements, 30% lower overall oxygen consumption and with the simultaneous removal of nitrate, reducing up to an estimate of 70% of carbon requirements for simultaneous N and P removal. Varying fractions of Denitrifying Polyphosphate Accumulating Organisms (DPAOs), from an average of 25% to 84%, were found in different plants. No correlation was found between the DPAO fractions and EBPR configuration, season nor the concentration of any of the microbial groups measured via quantitative fluorescence *in situ* hybridisation. These included Type I and Type II *Ca.* Accumulibacter and Glycogen Accumulating Organisms, suggesting that chemical batch tests are the best methodology for quantifying the potential of anoxic P removal in full-scale WWTPs.

KEYWORDS

Denitrification, DPAOs, EBPR, Enhanced Biological Phosphorus Removal, Full-scale, Nutrient removal

INTRODUCTION

Enhanced Biological Phosphorus Removal (EBPR) is a widely adopted technology for treating wastewater including the efficient removal of phosphorus. It addresses the growing concern of the environmental impacts caused by excessive nutrients in water bodies, known as eutrophication. This technology is based on alternating anaerobic and aerobic conditions to encourage the uptake of "luxury" phosphorus (P) by organisms who thrive under these conditions: Polyphosphate Accumulating Organisms (PAOs). There are many possible variations of the conventional configuration (Anaerobic/Aerobic or A/O), including the addition of one or multiple anoxic stages to achieve denitrification with the simultaneous removal of P (Sedlak 1991). Denitrification is achieved through the action of multiple groups of heterotrophic microorganisms, including PAOs (DPAOs), who can use nitrogen compounds (nitrate and/or nitrite) as electron acceptors. Theoretically and as shown in lab-scale studies, anoxic phosphorus removal is advantageous since it allows simultaneous P and nitrate removal on the same carbon source thus reducing oxygen requirements, as well as sludge production (Kuba et al. 1996a). However, whilst the process is well-established, very limited stoichiometric, kinetic and microbiological data is available on the capacity of full-scale EBPR plants to achieve simultaneous denitrification and phosphorus removal. Considering the recent advances provided by many studies on the identification of potential DPAOs (e.g., Carvalho et al. 2007; Flowers et al. 2009) and on their observed kinetic and stoichiometric parameters in enriched populations at lab-scale (e.g., Lanham et al. 2011; Rubio-Rincón et al. 2017; Ribera-Guardia et al. 2016; Saad et al. 2016), it is timely to investigate how this knowledge translates into the characterisation of full-scale communities.

PAOs and their known competitors, Glycogen Accumulating Organisms (GAOs), often also present in EBPR systems, have both been shown to denitrify. Within these organism groups, evidence has shown diversity within PAOs and GAOs in their denitrification pathways, from full denitrification from nitrate and/or nitrite to no denitrification. Early studies with limited microbial characterisation suggested two fractions of PAOs: DPAOs, able to remove phosphate in anoxic conditions (including the use of nitrate and nitrite), and non-DPAOs, only able to remove phosphate using oxygen as electron acceptor (Kerrn-Jespersen et al. 1993). Further studies, in particular of organisms belonging to the genus *Ca.* Accumulibacter, the most well-characterised PAO, showed different results regarding the denitrification capabilities of PAOs with nitrate and nitrite. Authors highlighted potential morphological (Carvalho et al. 2007), taxonomic (Flowers et al. 2009) and genetic (García-Martín et al. 2006) differences that may explain different phenotypes. Fundamentally there are still two viable hypotheses: i) the existence of separate phenotypes including non-DPAOs, nitrite-DPAOs (mostly correlated with Accumulibacter clade II), and nitrate-DPAOs (mostly correlated with Accumulibacter clade I) (Flowers et al. 2009, Lanham et al. 2011); ii) only one phenotype exists, nitrite-DPAOs, and the observed conversion of nitrate to nitrite in different studies is provided via flanking populations (e.g., Saad et al. 2016; Rubio-Rincón et al. 2017). *Tetrasphaera*-PAOs have been less studied. However, based on data of four isolates, they were shown to possess the genes for the reduction of nitrate to nitrite and some isolates (*T. japonica* and *T. elongata*) were shown to use nitrate and nitrite as electron acceptors (Kristiansen et al. 2012). Nevertheless, the amount of anoxic P uptake determined by a denitrifying *Tetrasphaera*-PAO enrichment has been found to be low (Marques et al. 2018). Regarding GAOs, as with Accumulibacter-PAOs, results indicate that taxonomic differences could result in different capacities to utilise nitrate and nitrite in both major studied organisms: *Ca.* Competibacter and *Defluviicoccus vanus*-related GAOs. For example, some Competibacter sub-groups could not denitrify, whilst others could use nitrate but not nitrite and some could use both (Kong et al. 2006). As

for *D.vanus*-related GAOs, Cluster 1 was shown to use nitrate whereas Cluster 2 could not and none could use nitrite (Wang et al. 2008; Burow et al. 2007). Due to the different findings as to the denitrification capacities of organisms involved in EBPR, a quantification of DPAOs could potentially be correlated with the abundance of Accumulibacter Type I PAOs or with the presence of GAOs.

Over time, different methods have been suggested to determine the fraction of DPAOs in mixed systems by quantification of the chemical transformations of phosphate and nitrogen. The assumption would be that under anoxic conditions, only PAOs able to use nitrate and/or nitrite (i.e. DPAOs) would remove phosphate, whereas under oxic conditions all PAOs would be able to remove phosphate. Thus, the rates or yields of P removal under anoxic conditions vs. aerobic conditions would be proportional to the ratio of DPAOs vs. PAOs. Initially, the ratio of the rate of P removal in parallel anoxic and aerobic batch tests was suggested (Wachtmeister et al. 1997). Subsequent improvements were proposed, first by Meinhold et al. (1999) and then by Oehmen et al. (2010), to use the ratio of phosphate removal to avoid kinetic biases. Based on these methods, sporadic estimations of DPAOs vs. non-DPAOs have been carried out in lab-scale (Oehmen et al. 2010; Wachtmeister et al. 1997), pilot scale (Meinhold et al. 1999; García-Usach et al. 2010) and full-scale (Wachtmeister et al. 1997; Bai et al. 2011) studies. However, there has not been a systematic assessment of the efficiency of anoxic P removal vs. aerobic P removal in full-scale systems using nitrate, coupled with a microbiological analysis that could attempt to link DPAO fractions to the presence of Accumulibacter Type I and Type II or GAOs.

Therefore, this study investigated the activity of DPAOs in five full-scale EBPR plants with different configurations and over a period of time. The data collected was able to provide an important insight into the variability of the kinetics and the microbial composition of simultaneous denitrification and P removal within different full-scale plants, as well as shed some light on some of the factors that might influence DPAO selection.

METHODS

Sample collection

Five full-scale EBPR plants, labelled PT1-PT2 and DK1-DK3 respectively, were included in this study: three conventional anaerobic/anoxic/aerobic systems (A_2O) (Beirolas WWTP, SIMTEJO, Lisbon, Portugal; Setubal WWTP, Águas do Sado, Setúbal, Portugal; Hjørring WWTP, Hjørring, Denmark) and two adapted Biodenitro configurations (Aalborg West and Aalborg East WWTP, Aalborg, Denmark). All plants were sampled at the end of the aerobic phase during winter conditions and the Portuguese plants were also sampled in summer conditions as detailed in Lanham et al. (2013).

Offline Batch Tests and Chemical Analysis

The activity of the PAO community in each sampled WWTP was tested using offline batch tests supplied with synthetic medium containing acetate as the main substrate. Nineteen tests were run as a 2-h anaerobic phase with the addition of an acetate (10-15 mg-C/L) and phosphate (30-40 mg-P/L) pulse. This slight variation is due to adjustments in the carbon and P concentration after the initial tests to ensure that a reasonable anaerobic carbon profile was determined and that P would always be in excess during the aerobic and anoxic phases. At the end of the anaerobic phase, the mixed liquor was split into two separate reactors, one aerobic (oxygen levels close to saturation) and one anoxic (pulse of 25 mg-N/L of nitrate) and run for approximately 4-6 h until PHA reserves were consumed and P removal stabilised, as proposed in Oehmen et al. (2010). In aerobic tests, the oxygen uptake rate (OUR) was measured by stopping the recirculation of mixed liquor into a respirometric side chamber and

measuring the decrease in dissolved oxygen (DO). In thirteen out of the nineteen experiments, a further aerobic phase was supplied after the anoxic phase to observe aerobic uptake of phosphate. pH and temperature were controlled at 7.0 ± 0.2 and 20 ± 1 ^{o}C respectively and dissolved oxygen was kept below 0.1 mg/L in anaerobic and anoxic conditions. Full details of sampling and operation are provided in Lanham et al. (2013).

The chemical transformations in the reactors were monitored by periodic sampling (typically every 5-20 min) for measurements of acetate, phosphate and ammonia in the supernatant and of PHA, glycogen and total phosphorus in the biomass. For each phase, initial and final samples of glycogen were measured in triplicate. Acetate was determined by high pressure liquid chromatography, phosphate was measured by the ascorbic acid colorimetric method and ammonia was measured by a gas sensing combination electrode. Lyophilised biomass samples were digested and used for the quantification of PHA and glycogen via gas and liquid chromatography respectively. Volatile suspended solids (VSS) were determined by the gravimetric method according to APHA (1995). Full details of the analytical methods are described in Lanham et al. (2013). Nitrate and nitrite were monitored in the supernatant in each of the reactors, with greater detail in the anoxic phase. For Portuguese tests and Danish Plant DK3, the analysis was done using ion chromatography according to Lanham et al. (2011) and for DK1 and DK2 using colorimetric quantitative test strips for nitrate and nitrite (Merck, Germany). The chemical concentrations are given in C-mol of active biomass (X) by subtracting the amount of glycogen and PHA from the VSS (biomass formula $CH_{1.84}O_{0.5}N_{0.19}$ (Zeng et al. 2003)).

Quantitative fluorescence *in situ* hybridisation (qFISH)

qFISH was performed on all mixed liquor samples using an Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) to quantify target organisms by their biovolume as detailed in Nielsen (2009) and Mielczarek et al. (2012). The organisms targeted are detailed in Lanham et al. (2013). In addition to this, Accumulibacter-PAO Type I and II were targeted using probes Acc-I-444 and Acc-II-444 by (Flowers et al. 2009a).

Estimation of carbon requirements

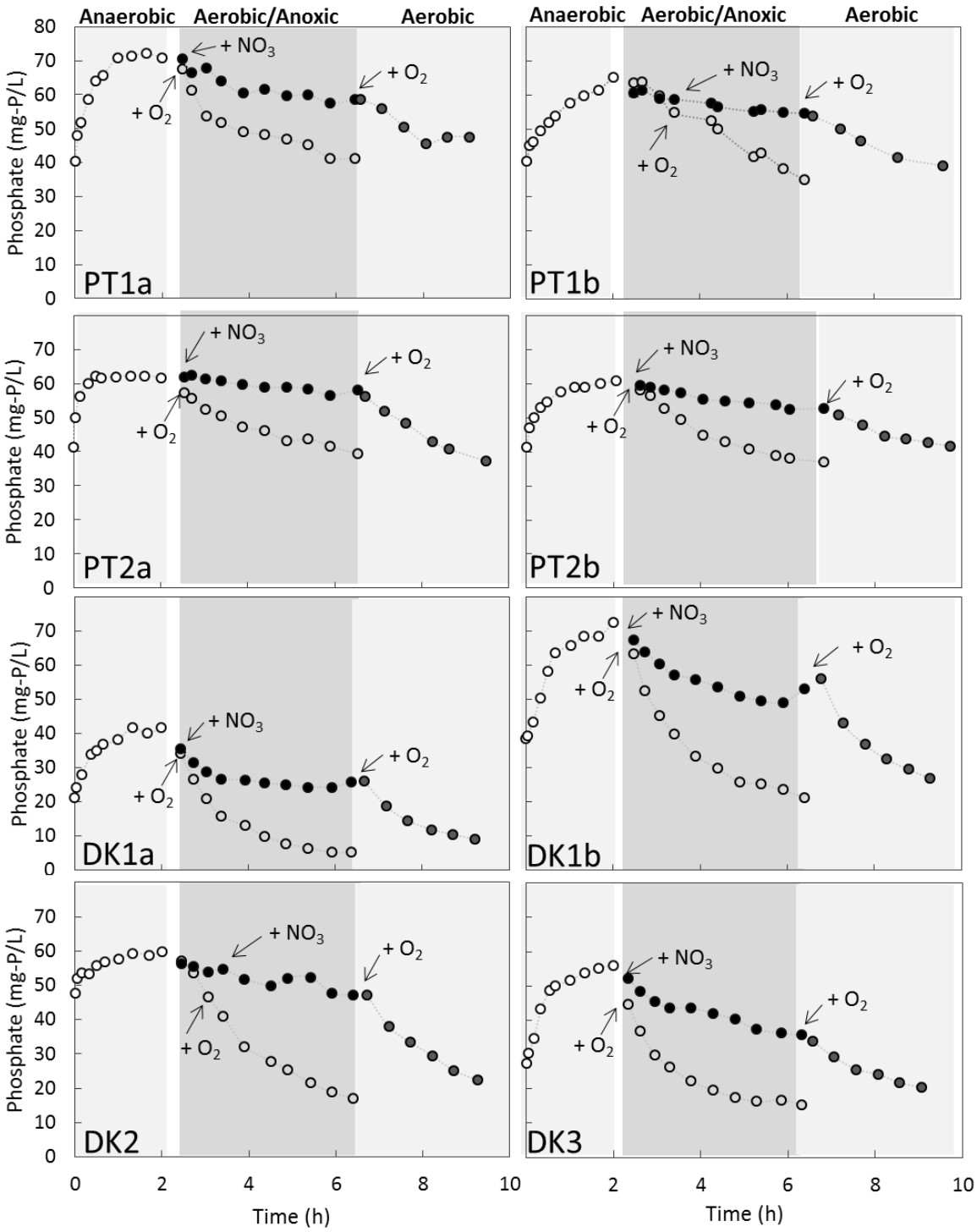
The carbon requirements were estimated in three different configurations: anoxic EBPR (A2O, this study), conventional nitrification-denitrification, and EBPR (A/O) with additional denitrification. These were calculated based on the level of nitrate reduction achieved in this study, i.e., removal of a minimum of 25 ppm-N of nitrate. An anoxic EBPR system, as presented in this study, would consume the COD provided in this study (i.e., 10-15 C-ppm of acetate equivalent to 27-40 ppm COD) and in addition to nitrate removal, could remove a minimum of 9 ppm-P. A nitrification-denitrification system, would require 72 ppm-COD for the removal of the same quantity of nitrate (assuming 2.86 mg-COD/mg NO3-N as per ASM1 model (Henze et al. 1987)). Finally, adding the two values above, the combination of an A/O EBPR with a separate nitrification-denitrification would require a total of 99-112 ppm-COD (COD for EBPR + COD for denitrification).

RESULTS AND DISCUSSION

Aerobic and Anoxic P removal capacity in different full-scale WWTPs

The first objective was to compare the anoxic and aerobic phosphate uptake in different full-scale EBPR plants using offline batch tests with acetate. After one single anaerobic phase, the mixed liquor was split into parallel aerobic and anoxic batch tests (addition of a nitrate spike). Examples of the phosphate cycling in the different experiments are given in **Error! Reference source not found.**. All

plants included EBPR configurations with anoxic conditions, hence it is not surprising that all mixed liquors were able to remove phosphate both aerobically and anoxically. On average, under anoxic conditions, DPAOs were only able to remove 35% of the P removed under aerobic conditions. These values varied from plant to plant, proportionally to the different DPAO fractions (Figure 3), with PT2 in the summer presenting the lowest anoxic P removal (13%) and DK1 the highest (46%). However, when supplied with an additional aerobic phase, the total P removed is 90 ± 3% of the P removed under only aerobic conditions (**Error! Reference source not found.**). This came at the expense of a marginal PHA and glycogen increase (109 ± 7 % and 106 ± 7 %) (**Error! Reference source not found.**). In terms of oxygen requirements, these depended on the fraction of DPAOs available and how much P was removed anoxically, but on average this configuration required only 70% of the oxygen needed for a full aerobic removal, as also observed by Kuba et al. (1996a) in a lab-scale system, with the added benefit of also removing approximately up to 25 ppm-N. This means simultaneously removing N and P at the expense of only 27-40 ppm COD instead of 99-112 ppm COD (c.f. methods section), i.e. a reduction in carbon requirements of approximately up to 70%, an estimate that is moderately higher to what was obtained by Kuba et al. (1996a) with lab-scale reactors (50%).



179 *Figure 1: Phosphate profiles in a sample of off-line batch tests across the 5 EBPR plants. The different data points were*
180 *obtained under anaerobic conditions (white circles) and then the sludge was partitioned into 2 parallel tests, one under*
181 *anoxic conditions (black circles) and one under aerobic conditions (light grey circles), and finally the anoxic sludge was*
182 *subjected to an aerobic phase (dark grey circles). Samples were run in triplicate and standard deviation was below 5%.*
183 *Anaerobic and aerobic profiles of other compounds are shown in Lanham et al. 2013.*
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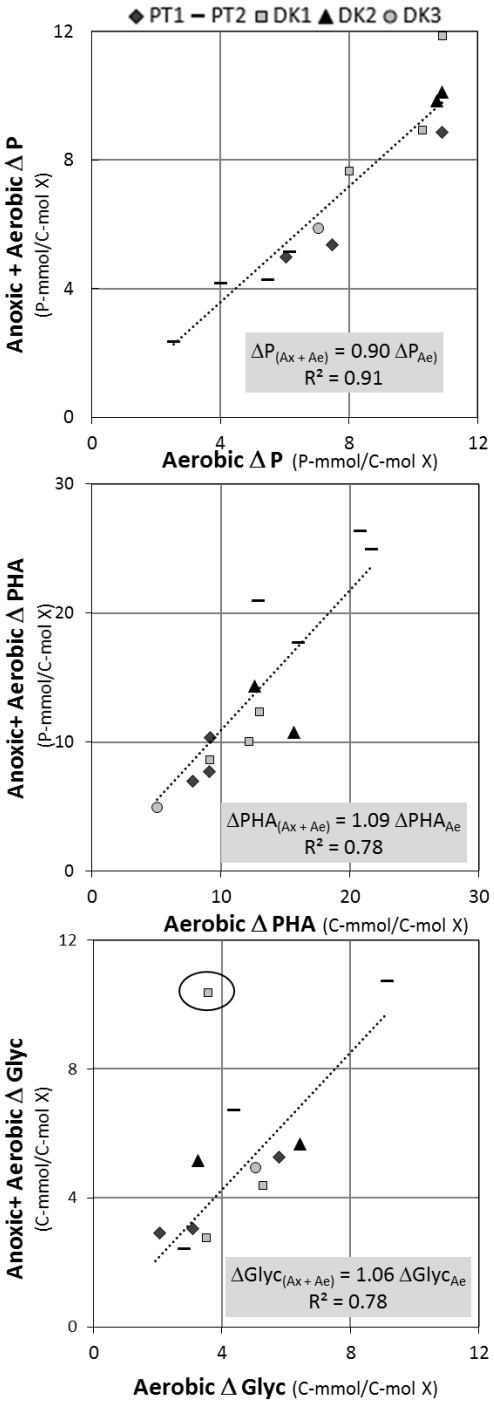

Figure 2: Correlation between phosphate (top), PHA (middle) and glycogen (bottom) uptake under aerobic conditions vs. anoxic + aerobic conditions in the different plants (PT1 – diamond, PT2 – dash, DK1 – square, DK2 – triangle, DK3 – circle). The circled result has been excluded from the linear regression, due to an extremely high measurement of glycogen in the anoxic phase that could suggest an experimental error.

In all batch tests, the rate of anoxic P removal (rP) was on average a third of the rate of aerobic P removal (Table 1). This is similar to results obtained by Bai et al. (2011), also with full-scale sludge. Once oxygen is introduced at the end of the anoxic phase, P uptake re-starts at a higher rate, but lower than the one observed when only an aerobic phase is supplied, as also observed by Kerrn-Jespersen and Henze (1993). Both anoxic and aerobic P-uptake are accompanied by the intracellular use of PHA

198 and glycogen replenishment. This suggests that PAOs are active under anoxic conditions with nitrate,
199 either because they can directly denitrify or because flanking populations are converting nitrate to
200 nitrite as proposed by Rubio-Rincón et al. (2017)), and this would be the rate limiting step as no nitrite
201 accumulation was observed (typically below detection limit of 1 ppm-N).

202 Under anoxic conditions, excluding the results from PT2, where a high number of GAOs was detected,
203 the yields of glycogen replenishment on intracellular PHA (Glyc/PHA) were within the ranges proposed
204 in previous metabolic models (Table 2). However, the yield of phosphate removal at the expense of
205 PHA (P/PHA) seemed more variable and higher than expected (Table 2). This could suggest the
206 presence of other PAOs not or less reliant on PHA and glycogen, such as for example *Tetrasphaera*-
207 PAOs, that would be able to contribute to some P removal (Marques et al. 2017). However, even if
208 *Tetrasphaera* has been detected in significant quantities in all samples in this study (*cf.* Lanham et al.
209 (2013)), they are the most abundant in plants DK1, DK2 and then PT1, which does not seem to align
210 with the calculated P/PHA yields, with the highest being in DK3 followed by PT1.

211 In terms of the ratios of P removed per electron acceptor (P/O_2 for oxygen and P/N for nitrate) (Table
212 1), the values are similar to the ones reported by Bai et al (2011) for full-scale experiments but
213 generally lower than previous values reported in literature for lab-scale enriched cultures both with
214 nitrate and nitrite as electron acceptor (Table 1). This implies a lower overall efficiency of full-scale
215 sludge to remove P in function of their electron acceptor. This could be a result of the lower
216 concentration of PAO organisms in the mixed liquor coupled with a residual maintenance activity of
217 other microbial communities relying on these electron acceptors. This emphasizes the importance of
218 measuring the aerobic and anoxic P removal in full-scale systems in addition to the fundamental
219 studies in lab-scale enriched communities.

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222 *Table 1: Stoichiometric and kinetic values related to P uptake under aerobic and anoxic conditions. Results shown are averages*
223 *for all sampling points (n = number of tests), standard deviation and range of values to illustrate the spread of data.*

		Aerobic		**Anoxic**		**Aerobic (after Anoxic)**	
		P/O_2	rP	P/N	rP	P/O_2	rP
		P-mol/O_2-mol	mg-P/gVSS.h	P-mol/N-mol	mg-P/gVSS.h	P-mol/O_2-mol	mg-P/gVSS.h
PT1 summer n_{AE} or n_{AX} = 4, n_{AX+AE} = 2		0.38 ± 0.03 (0.35 – 0.42)	8 ± 3 (3.7 – 11.4)	0.25 ± 0.08 (0.14 – 0.32)	2.9 ± 0.8 (1.7– 3.6)	0.15 ± 0.04 (0.12 – 0.18)	2.9 ± 0.8 (2.3 – 3.8)
PT1 winter n_{AE} or n_{AX} = 2, n_{AX+AE} = 1		0.40 ± 0.06 (0.35 – 0.44)	3 ± 1 (2.5 – 4.0)	0.17 ± 0.02 (0.15 – 0.18)	1.0 ± 0.5 (0.7 – 1.4)	ND	2.0 (n=1, 2.0)
PT2 summer n_{AE} or n_{AX} = 2, n_{AX+AE} = 2		0.20 ± 0.08 (0.14 – 0.25)	2.0 ± 0.2 (1.9 – 2.1)	0.05 ± 0.01 (0.04 – 0.06)	0.6 ± 0.3 (0.4 – 0.9)	0.2 ± 0.1 (0.13 – 0.29)	1.9 ± 0.2 (1.7 – 2.0)
PT2 winter n_{AE} or n_{AX} = 2, n_{AX+AE} = 2		0.25 ± 0.04 (0.22- 0.27)	2.5 ± 0.2 (2.4 – 2.7)	0.16 ± 0.02 (0.15- 0.17)	0.8 ± 0.1 (0.7 – 0.8)	0.21 ± 0.04 (0.18 – 0.23)	1.8 ± 0.1 (1.7 – 1.9)
DK1 winter n_{AE} or n_{AX} = 4, n_{AX+AE} = 3		0.40 ± 0.07 (0.29 – 0.45)	7.2 ± 0.7 (6.4 – 8.0)	ND	3 ± 2 (1.6 – 5.6)	0.3 ± 0.1 (0.27 – 0.45)	4 ± 1 (2.9 – 5.5)
DK2 winter n_{AE} or n_{AX} = 3, n_{AX+AE} = 2		0.40 ± 0.08 (0.31 – 0.47)	5 ± 1 (4.1 – 6.0)	ND	1.2 ± 0.2 (1.0 – 1.4)	0.43 ± 0.0 (0.42 - 0.43)	4.2 ± 0.9 (3.6 – 4.9)
DK3 winter n_{AE} or n_{AX} = 2, n_{AX+AE} = 1		0.29 ± 0.08 (0.24 – 0.35)	6.1 ± 0.0 (6.1 – 6.1)	0.26 ± 0.08 (0.20– 0.31)	2.0 (n=1, 2.0)	0.24 (n=1, 0.24)	2.0 (n=1, 2.0)
Full-scale, HAc, NO^{3-}	Kuba et al. (1997) (n=1)	-	13	-	6	-	-
	Bai et al. (2011) (n=3)	-	2.00 – 3.85	0.05 – 0.3	0.54 – 2.22	-	-
Lab scale, HAc, NO^{3-}	Kuba et al. (1993)	0.91	29 - 46[a]	0.94	29 - 46[a]	-	-
	Carvalho et al. (2007)	-	-	0.6[b]	0.6 – 8.4	-	-
Lab scale, HPr, NO^{3-}	Carvalho et al. (2007)	-	-	0.82[b]	3.1 – 19.5	-	-
	Guisasola et al. (2009)	-	-	1.6[b]	0.6 - 2.2	-	-
Lab scale, HAc, NO^{2-}	Saad et al. (2016)	1.38	-	0.70	17.1	-	-
Lab scale, HPr, NO^{2-}	Saad et al. (2016)	1.28	-	0.46	12.4	-	-
	Guisasola et al. (2009)	-	-	0.29[b]	3.7 - 7.13	-	-

[a]calculated by converting gSS to gVSS using 0.65-0.70 gVSS/gSS
[b]calculated from anoxic rates of P-uptake/N-uptake

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Table 2: Experimental results for offline anoxic batch tests (n=19) followed by an aerobic phase (n= 13). Results shown are averages rounded to one decimal (n = number of tests) with a standard deviation and the range of values to illustrate the spread of the data.

	Anoxic + Aerobic Experimental Results			
	Anoxic Phase		**Aerobic Phase**	
	P/PHA P-mol/C-mol	Glyc/PHA C-mol/C-mol	P/PHA P-mol/C-mol	Glyc/PHA C-mol/C-mol
PT1 summer (n_{AX} = 4, n_{AX+AE} = 2)	0.7 ± 0.2 (0.53 – 0.83)	0.7 ± 0.5 (0.31 – 1.26)	1.7 ± 0.7 (1.20 – 2.17)	0.9 ± 0.2 (0.77 – 1.06)
PT1 winter (n_{AX} = 2, n_{AX+AE} = 1)	0.7 ± 0.5 (0.26 – 1.04)	0.3 ± 0.1 (0.28 – 0.40)	0.9 (n=1, 0.86)	0.3 (n=1, 0.28)
PT2 summer (n_{AX} = 2, n_{AX+AE} = 2)	0.0 ± 0.0 (0.03 – 0.05)	0.1 (n=1, 0.13)	0.3 ± 0.0 (0.31 – 0.32)	0.1 (n=1, 0.10)
PT2 winter (n_{AX} = 2, n_{AX+AE} = 2)	0.2 ± 0.0 (0.19 -0.25)	0.1 ± 0.0 (0.05 – 0.10)	0.2 ± 0.0 (0.15 – 0.19)	0.5 ± 0.1 (0.38 – 0.57)
DK1 winter (n_{AX} = 4, n_{AX+AE} = 3)	0.7 ± 0.2 (0.59 – 1.00)	0.5 ± 0.6 (0.02 – 1.36)	1.3 ± 0.2 (1.05 – 1.42)	0.7 ± 0.2 (0.52 – 0.90)
DK2 winter (n_{AX} = 3, n_{AX+AE} = 2)	0.7 ± 0.3 (0.50 – 1.05)	0.3 ± 0.1 (0.24 – 0.39)	0.9 ± 0.1 (0.81 – 0.90)	0.5 ± 0.2 (0.36 – 0.59)
DK3 winter (n_{AX} = 2, n_{AX+AE} = 1)	1.0 ± 0.1 (0.92 – 1.07)	0.5 ± 0.6 (0.06 – 0.88)	1.4 (n=1, 1.38)	1.0 (n=1, 1.04)
PAO model	0.30[b]	0.41[b]	0.41[a]	0.42[a]

[a] According to Smolders et al. (1995), [b] According to Kuba et al. (1996b)

Calculation of DPAO fraction

The experimental results obtained from the offline batch tests provided a means to determine the fraction of DPAOs (f_{DPAO}) and non-DPAOs (f_{n-DPAO}) over total Accumulibacter-PAO in each of the sampling points (Figure 3). For this purpose, a modification of the method proposed in Oehmen et al. (2010) was used (Equations (1(2), where f_{DPAO} is assumed to be correlated with the quantity of P removed under anoxic conditions with nitrate as the electron acceptor vs. the quantity of P removed under aerobic conditions, in parallel batch tests containing the same sludge. The modification accounts for nitrate having a lower energetic efficiency than oxygen as an electron acceptor due to a lower ATP production during the oxidative phosphorylation mechanism (Kuba et al. 1996b; Murnleitner et al. 1997). The phosphorus uptake in anoxic conditions was corrected using the P/O ratios (δ_{aer}, and δ_{anox}), i.e. the amount of ATP produced per oxidised $NADH_2$ during phosphorus uptake under aerobic and anoxic conditions respectively. Smolders et al. (1994) proposed that δ_{aer} is 1.85 and Kuba et al. (1996b) proposed that δ_{anox} is 1.

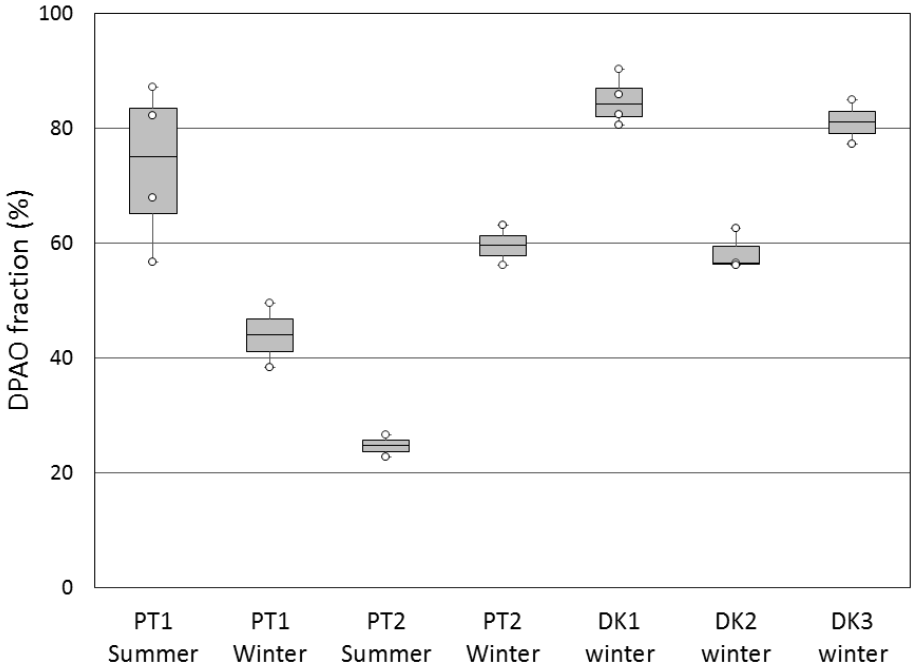

Figure 3: Boxplot representing DPAO fractions (white circles) in each plant and sampling period (winter and summer).

The highest denitrification activity was found in DK1, with a median of 84% of the PAO population being DPAOs, and the lowest (25%) was found in PT2 during the summer sampling. In the plants with seasonal sampling, there were quite significant differences in DPAO activity, suggesting that this capacity can fluctuate quite significantly (from medians 75% to 44% in PT1 and 25% to 60% in PT2 for summer and winter samplings respectively).

$$f_{DPAO} + f_{n-DPAO} = 1 \quad (1)$$

$$f_{DPAO} = \frac{\Delta P_{anox}}{\Delta P_{aer}} \times \frac{\delta_{aer}}{\delta_{anox}} \quad (2)$$

Correlation between microbial composition and DPAO activity

All samples contained 2-7% of *Ca*. Accumulibacter. Total concentrations of putative *Tetrasphaera*-PAOs were higher, ranging from 15-30%, however considering most are not likely to consume acetate, their activity should not be expressed in the experimental results. Finally, Competibacter and *Defluviicoccus*-related GAO concentrations were far more variable ranging from 1 to 6%. 7 out of the 19 samples contained either only Competibacter-GAOs (n=1), only *Defluviicoccus*-related GAOs (n=4) or both (n=2) in concentrations above 0.3%. These results are detailed in Lanham et al. (2013).

The ability of PAOs to denitrify from nitrate has been suggested to be linked with diversity within different types of Accumulibacter (Flowers et al. 2009), with some evidence suggesting that Type I would be able to denitrify from nitrate, whereas Type II only from nitrite, although Saad et al. (2016) has also reported a population of Accumulibacter clade IC not able to denitrify from nitrate. However in this study, there does not seem to be any correlation between the biovolume of Type I

Accumulibacter nor its fraction over the total PAO biovolume and the capacity to remove P under anoxic conditions in each of the plants (n = 19, R^2 is 0.27 and 0.06 respectively). Similarly, no correlation was found for the biovolume of Accumulibacter Type II nor its fraction over total PAO biovolume (n = 19, R^2 are both 0.00).

Average results for the q-FISH quantification of Type I and Type II Accumulibacter biovolumes in the different samples across the five tested WWTPs are shown in Table 3. It is important to note that these two probes do not necessarily cover all the diversity within Accumulibacter-PAOs (Flowers et al. 2009) and there might even be some overlap, as can be seen by calculating the ratio of the sum of Type I and Type II over total PAO determined by the PAOmix FISH probe (Table 3). In fact, for many of the samples, these two probes only covered approximately 50% of the organisms targeted by the probe PAOmix. In some others, their value was higher than PAOmix, which could indicate some Accumulibacter over-estimation using the PAOmix probe due to the inclusion of *Propionivibrio aalborgensis*, a putative GAO, as recently presented by Albertsen et al. (2016).

It has also been suggested that PAOs would only be able to denitrify from nitrite and that they would rely on flanking populations to produce nitrite from nitrate (Rubio-Rincón et al. 2017) and potentially on GAO-related populations. However, from the samples collected, there was also no observable correlation between the presence of GAOs and any increased phosphate removal under denitrification conditions. This applies for Competibacter-GAOs (n=19, R^2 is 0.00, 0.00 and 0.05 for the relationship between DPAO fraction and Competibacter biovolume, fraction of Competibacter over PAO biovolume and over total GAO biovolume, respectively) and for *Defluviicoccus*-related GAOs (n=19, R^2 is 0.20 and 0.30 for the relationship between DPAO fraction and *Defluviicoccus* biovolume and fraction of *Defluviicoccus* over total PAO biovolume, respectively). In fact, the samples with the highest concentration of GAOs, observed in plant PT2, resulted in the lowest P removal in anoxic conditions.

In conclusion, even if a wide range of DPAO activity has been found, varying seasonally and from plant to plant, this could not be explained by the microbiological results in terms of the presence of Accumulibacter Type I or II, Competibacter or any other detected GAOs. Therefore, chemical batch tests are the best methodology to assess a plant's potential of simultaneous P and N removal.

Table 3: Microbial composition of sampling points in each WWTP in terms of Accumulibacter *type I and type II. Values given are averages with standard deviations, plus the range of experimental values.*

	Type I Accumulibacter (% biovolume Acc-I-444)	Type II Accumulibacter (% biovolume Acc-II-444)	Ratio Type I + Type II *vs.* Total Accumuli.
PT1 summer (n = 4)	0.5 ± 0.1 (0.39 – 0.66)	0.7 ± 0.0 (0.65 – 0.71)	0.4 ± 0.1 (0.30 – 0.53)
PT1 winter (n = 2)	1.5 ± 0.1 (1.41 – 1.59)	1.5 ± 0.1 (1.41 – 1.59)	0.9 ± 0.0 (0.90 – 0.94)
PT2 summer (n = 2)	0.4 ± 0.1 (0.31 – 0.49)	1.1 ± 0.4 (0.84 – 1.36)	0.5 ± 0.0 (0.47 – 0.51)
PT2 winter (n = 2)	2.7 ± 0.0 (2.62 – 2.68)	2.8 ± 0.0 (2.80 – 2.84)	1.4 ± 0.4 (1.14 – 1.65)
DK1 (n = 4)	1.6 ± 0.2 (1.41– 1.88)	1.7 ± 0.4 (1.22 – 2.08)	1.0 ± 0.2 (0.79 – 1.25)
DK2 (n = 3)	1.9 ± 0.5 (1.26 – 2.28)	1.7 ± 0.4 (1.29 – 2.09)	0.5 ± 0.2 (0.37 – 0.68)
DK3 (n = 2)	1.5 ± 0.1 (1.39 – 1.55)	1.3 ± 0.1 (1.18 – 1.37)	0.6 ± 0.0 (0.59 – 0.62)

CONCLUSIONS

This work systematically assessed the capacity of five full-scale EBPR plants to remove phosphorus under anoxic and aerobic conditions, as well as the fraction of DPAO organisms in each of the sampling points. The results of a total of 19 independent tests showed that all plants were able to remove phosphate anoxically, even if only partially (35%) and at lower rates than in aerobic conditions. However, in a configuration with anoxic followed by aerobic conditions, approximately the same phosphate removal was achieved (90%), with only 70% of the oxygen requirements, and given the simultaneous nitrate uptake, reducing up to 70% of the carbon requirements for N and P removal. DPAO fractions varied across plants and in the same plant over time, from 25% (PT2) to 84% (DK1). There was no correlation found between the microbial composition of the samples, especially in terms of Accumulibacter Type I or Competibacter as suggested in the literature, with the abundance of DPAOs through anoxic and aerobic batch tests. These results suggest that chemical tests are still the best method to determine the performance of a full-scale EBPR system under anoxic conditions and that further work still has to ascertain which microbial groups are responsible for anoxic P removal from nitrate.

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